Gamma-Aminobutyric Acid-containing Terminals Can Be Apposed to Glycine Receptors at Central Synapses

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Abstract. The distributions of terminals containing gamma-aminobutyric acid (GABA) and of endings apposed to glycine receptors were investigated cytochemically in the ventral horn of the rat spinal cord. For this purpose, a polyclonal antibody raised to recognize glutamic acid decarboxylase (GAD), a synthetic enzyme for GABA, and three monoclonal antibodies (mAb's) directed against the glycine receptor were used. Double immunofluorescence showed that, surprisingly, GAD-positive terminals are closely associated in this system with glycine receptors at all the investigated cells, most of which were spinal motoneurons. Furthermore, double labeling was performed with immunoenzymatic recognition of GAD and indirect marking of mAb's with colloidal gold. With this combined approach, it was found, at the electron microscopic level, that (*a*) all GAD-positive terminals are in direct apposition with glycine receptors while, on the other hand, (*b*) not all glycine receptors are in front of GABA-containing boutons.

This result is not due to a cross-reactivity of mAb's with GABA receptors as shown by using as a control synapses known to use GABA as a neurotransmitter in the cerebellar cortex. Indeed, no glycine receptor immunoreactivity was detected on Purkinje cells facing basket axon terminals. However, Purkinje neurons can express glycine receptor immunoreactivity at other synaptic contacts.

Assuming that the presence of postsynaptic receptors for glycine indicates that this amino acid is used for neurotransmission at a given synapse, our results strongly support the notion that GABA and glycine, two classical inhibitory transmitters, coexist at some central connections. However, such is not always the case; in the cerebellum, Golgi terminals impinging on the dendrites of granule cells are either GAD-positive or face glycine receptors, in a well-segregated manner.

As shown with classical electrophysiological techniques, glycine and gamma-aminobutyric acid (GABA)[1] receptors can coexist on single neurons, both in culture (Ransom et al., 1977; Barker and Ransom, 1978; Choquet and Korn, 1986) and in vivo (Werman et al., 1968; Diamond and Roper, 1973; Faber and Korn, 1980); and patch-clamp studies of spinal cord cells have shown that these receptors can coexist within a 5–20-μm area of membrane (Hamill et al., 1983). However, although both glycine and GABA act by increasing chloride conductances (Werman et al., 1968; Krnjevic and Schwartz, 1967; Curtis et al., 1968; Ransom et al., 1977; Barker and Ransom, 1978) they bind to specific receptors (Werman et al., 1967; Curtis and Johnston, 1974; Krnjevic, 1974; see also Peck, 1980) and their activated channels often have different kinetic properties (Hamill et al., 1983).

From a morphological point of view, several specific markers are now available to localize these neurotransmitters or their receptors; two of them can be used in combination to mark both sides of synapses. (*a*) The first, aimed more specifically at identification of GABA-containing terminals, uses antibodies directed against glutamic acid decarboxylase (GAD) (Saito et al., 1974), a synthetic enzyme for this transmitter. Results with this approach are quite compelling since the cellular and subcellular distributions of GAD were quite the same throughout the whole central nervous system (MacLaughlin et al., 1975; Ribak et al., 1977), as those obtained with local uptake of ^{3}H-GABA (Iversen and Schon, 1973) and with an antibody against GABA itself (Somogyi et al., 1985). The presence of GAD is therefore considered as one of the best indicators of neurons that involve GABA for neurotransmission (Oertel et al., 1984) and for which the word GABAergic is classically used. (*b*) The second uses mAb's raised against various determinants of the glycine receptor, including the 93-kD-associated protomer (Pfeiffer et al., 1984). These immunoglobulins have been shown to allow probing the distribution and subcellular organization of glycine receptor molecular complex in the spinal cord (Triller et al., 1985) and cochlear nucleus of the rat (Altschuler et al., 1986) as well as in the teleost Mauthner cell (Triller et al., 1986). This technique is obviously more precise than, but complementary to, earlier ones which, nevertheless, helped to localize glycinergic terminals, particularly in the

1. *Abbreviations used in this paper*: GABA, gamma-aminobutyric acid; GAD, glutamic acid decarboxylase; GAM, goat anti-mouse; RAM, rabbit anti-mouse; SAM, sheep anti-mouse.

spinal cord, by autoradiography of glycine uptake (Hökfelt and Ljungdahl, 1971; Matus and Dennisson, 1971, 1972; Wilkin et al., 1981; Pourcho et al., 1980) or by comparing high affinity transport (Aprison and Daly, 1978; Logan and Snyder, 1972) and receptor binding (Young and Snyder, 1974; Zarbin et al., 1981; Rotter et al., 1984).

With polyclonal antibodies directed against GAD and monoclonal immunoglobulins directed against glycine receptors, questions pertinent to the physiological similarities and to the coexistence of the two receptors on the same cells could be addressed. More specifically, (*a*) are the two systems segregated, or rather intermingled; and (*b*) do ultrastructurally stereotyped inhibitory terminals have the same immunocytochemical staining properties? As a first step, the structure most commonly associated with glycinergic mechanism (namely, the vertebrate spinal cord) was used for this purpose; in contradiction to all expectations, we found that some GAD-positive terminals faced glycine receptors (although many postsynaptic differentiations bearing the latter were free of such innervation). This observation raised another issue, that of a possible cross-reaction between the mAb's for glycine and GABA receptors or their associated proteins. Use of the cerebellum showed that this was not the case and furthermore facilitated the detection of glycine receptors in regions where their presence had not been suspected so far. Again, some GAD-containing stellate terminals did face glycine receptors, but at the level of the granular layer Golgi axons were either GAD-positive or apposed to glycine receptors, thus indicating that at the level of the granular layer the two systems are distinct.

Thus, there exists a diversity of the immunocytochemical organization of vertebrate inhibitory synapses, the functional meaning of which is still unclear.

Materials and Methods

Antibodies Used

Three mAb's, which have been prepared (Pfeiffer et al., 1984) against glycine receptors that were previously solubilized in the presence of detergent (Pfeiffer and Betz, 1981) and purified on a strychnine affinity column (Pfeiffer et al., 1982), were used as previously described (Triller et al., 1985). Two of them, mAb's GlyR5a and GlyR2b, react with a single protomer, the 93-kD and the 48-kD subunits, respectively (the strychnine being bound irreversibly to the last one). The third, GlyR7a, reacts with the 93-kD subunit, and, to a lesser extent, with the 48-kD protomer (Pfeiffer et al., 1984).

GABA-containing terminals were charted with a high sensitivity and high affinity antiserum directed against GAD, furnished to us by Dr. Tappaz (INSERM U171, Lyon, France) and produced in sheep (Oertel et al., 1981).

Fixation Procedures

Rats (Fisher 344) were anesthetized with pentobarbital (30 mg/kg) and perfused intracardiacally for 30 min with solutions which best allow the antibodies to be visualized. For light microscopy, 4% paraformaldehyde in 0.12 M phosphate buffer at a pH of 7.4 was preferred. For electron microscopy, the composition was 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer, followed by the former alone (this concentration of glutaraldehyde is low, as it is usually the case for this type of study, in order to minimize the loss of antigenicity, and to reduce the extent of protein cross-linking [Glauert, 1975]).

Immunofluorescence

(*a*) For the localization of the different mAb's, unfixed cervical spinal cord was sectioned (5 μm thick) with a cryostat. Slices were incubated for 1 h with mAb GlyR2b diluted (1:200) in PBS, washed with the same buffer, treated with paraformaldehyde (4%) for 10 min, then washed again with buffer and finally incubated with TRITC coupled to sheep anti-mouse (SAM-TRITC [1:200 in PBS]; Cappel Laboratories, Cochranville, PA). Subsequently, the preparation was treated (15 min) with rabbit anti-mouse (RAM [1:20]; Nordic Immunological Laboratories, Tilburg, The Netherlands) in order to block antigenic determinants on the Fc fragment of the first antibodies which would have otherwise remained unoccupied by the SAM-TRITC. They were then incubated for another hour with either mAb GlyR5a or GlyR7a, and for 30 min with a goat anti-mouse Ig coupled to FITC (GAM-FITC [1:200 in PBS]; Nordic Immunological Laboratories).

(*b*) For simultaneous visualization of GAD and glycine receptors, spinal cord and cerebellum were washed in PBS and freeze sectioned (30 μm thick) in slices that were incubated in a mixture of first antibodies: GAD (1:2,000) and mAb GlyR5a (1:200), or GAD (1:2,000) and mAb GlyR7a (1:200). The sections were then sequentially incubated with biotinylated horse anti-mouse (1:200, 2 h; Vector Laboratories, Inc., Burlingame, CA) and fluorescein-streptavidine (1:200, 2 h; Amersham International, Amersham, UK) for revelation of the mAb's. They were finally reacted with TRITC-donkey anti-goat (1:200, 2 h; Nordic Immunological Laboratories) for visualization of the GAD.

Immunoelectron Microscopy

For electron microscopy, the spinal cord and/or the cerebellum were sectioned with a Vibratome (Oxford Instruments), 50 μm thick, in ice-cold PBS, and incubated with a mixture of antibodies against glycine receptors and GAD (at the same concentration as above), overnight at room temperature.

Immunoperoxidase Staining. The "Vectastain" (Vector Laboratories, Inc.) immunoperoxidase staining procedure was used as follows. After incubation with the antibodies, the sections were kept for 1 h in biotinylated donkey anti-goat IgG (1:200, Amersham International) associated, in case of double immunoenzymatic staining, with horse anti-mouse IgG (1:200, Vector Laboratories, Inc.) also biotynilated: specimens were subsequently washed, and finally incubated for 2 h with the avidin and biotinylated reagent, prepared to form ideal complexes for immunoperoxidase staining ("Vectastain ABC," reagent, Vector Laboratories, Inc.). The enzymatic reaction was carried out using 0.5% diaminobenzidine tetrahydrochloride in 0.05 M Tris buffer (pH 7.4). The reaction time (from 5 to 20 min) was controlled under the light microscope.

Immunogold Labeling. Penetration of gold particles was achieved during fixation by hypoosmotic shock (de Camilli et al., 1983; Triller et al., 1985) using the same fixative as above, except that the 0.12 M buffer was replaced by 5 mM phosphate buffer (de Camilli et al., 1983). The cervical cord was then immediately removed and sliced with a Vibratome: the sections were kept for 16 h with the selected mAb (GlyR5a or GlyR7a), washed in PBS, dipped in RAM for 2 h (1:200, Nordic Immunological Laboratories, Inc.), and treated during 6 h with protein A absorbed on 5.5 ± 1.4-nm colloidal gold particles (Roth, 1982). For simultaneous immunoenzymatic visualization of the antigens, the slices were treated for another 12 h with anti-GAD antibody, and revealed with the biotin-avidine peroxidase technique, as described above.

All EM observations were carried out on sections from the first 5–10 μm under the surface of the block, where the two types of labeling were present, penetration of the antibodies and other reactants being less consistent below this level.

Results

The first series of observations reported here were made at the level of the ventral horn of the spinal cord of the rat in lamina VIII and IX of Rexed (1954), in which neurons with large somata can be identified as motoneurons. Controls and further data were obtained from the cerebellar cortex.

Distribution of Determinants Recognized by mAb's against Glycine Receptors

All the mAb's did not recognize the same subunits of the glycine receptors and therefore we had to determine whether the different epitopes are all present at the same synapses. Since the determinant recognized by mAb GlyR2b was destroyed by classical aldehyde fixation (Triller et al., 1985), this goal was achieved on unfixed material.

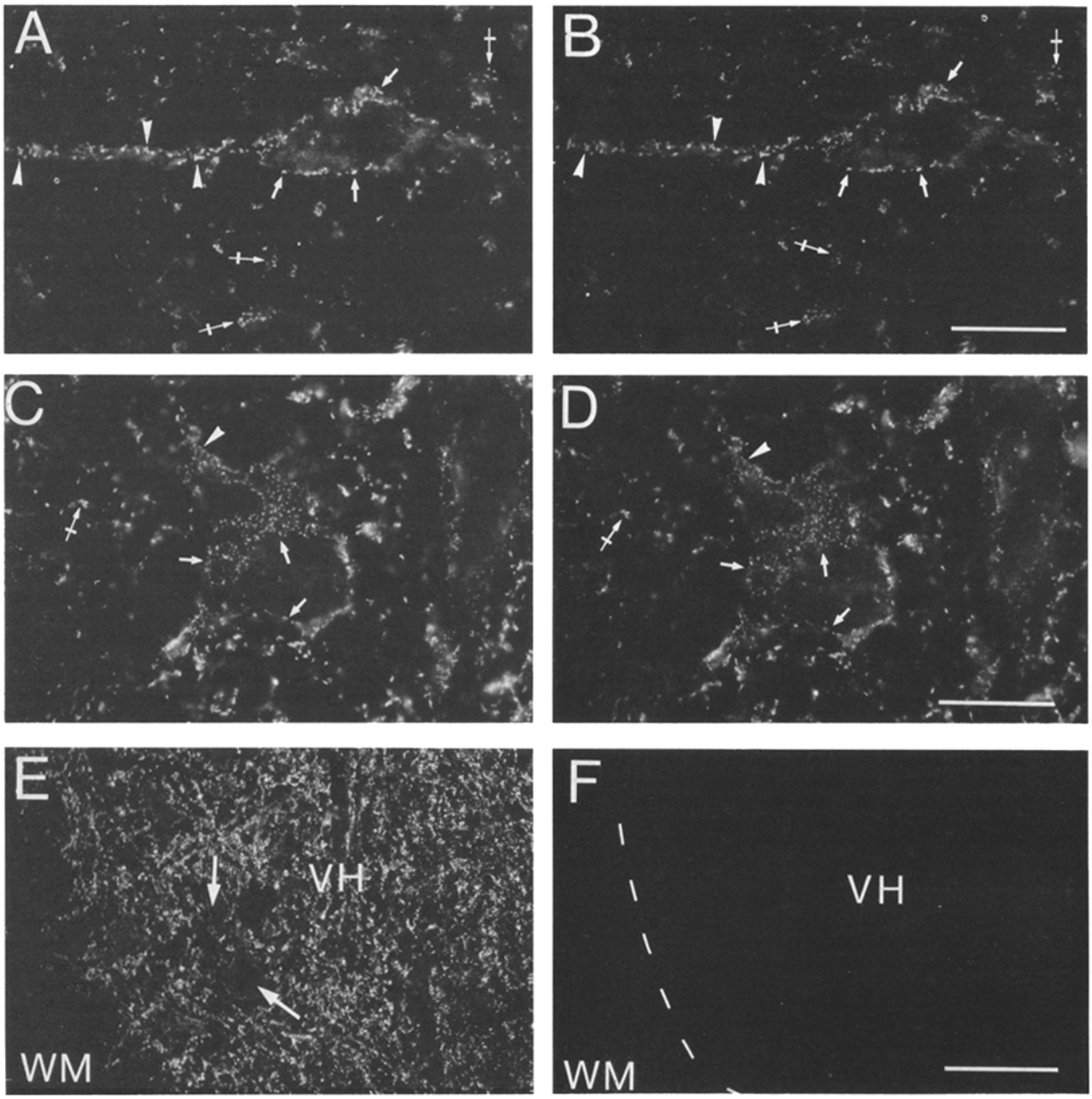


Figure 1. Proximity of determinants recognized by the three mAb's (GlyR2b, 7a, and 5a) in the ventral horn of the rat spinal cord. Cryostat sections were fixed with paraformaldehyde (4%) after incubation with mAb GlyR2b. (*A* and *B*) Indirect immunofluorescence showing discrete patches over the surface of the soma (*arrows*) and dendrites (*arrowheads*) of large multipolar neurons. Note that the patterns of labeling are identical on transverse sections sequentially treated with mAb GlyR2b, SAM-TRITC, RAM, GlyR5a, and GAM-FITC when examined for rhodamine (*A*) or fluorescein (*B*). (*C* and *D*) Colocalization of GlyR2b (*C*) and GlyR7a (*D*) demonstrated by double labeling, as above. Bright patches on the background (*crossed arrows*) are presumably due to staining of dendritic synapses. (*E* and *F*) Control sections labeled with GlyR2b only, and treated with SAM-TRITC, RAM, and GAM-FITC. (*E*) With filters for rhodamine, the ventral horn (*VH*) and some large cells (*arrows*) within it are intensely labeled while the white matter (*WM*) is not. (*F*) In contrast with filters for fluorescein, there is no staining as mAb GlyR2b is blocked (see Materials and Methods for explanation). The limit between the ventral horn and the white matter is delineated by a dotted line. Bars: (*A–D*) 10 μm; (*E* and *F*) 50 μm.

When the sections were double-labeled with both GlyR2b and GlyR5a, and observed with appropriate filters, fluorescent patches were found to be discontinuous at cell surfaces (Fig. 1, *A* and *B*). A comparison of the fluorescence due to the two antibodies showed that patches detected with either method matched exactly. A similar patchy distribution and matching were obtained when GlyR2b and GlyR7a were used as primary antibodies (Fig. 1, *C* and *D*). On the other hand, controls without one of the mAb's led to the absence of the corresponding fluorescence. In our experiments, the mAb GlyR2b was recognized with SAM-TRITC; in this case, all the Fc fragments of the mAb were probably not occupied by the anti-mouse immunoglobulin. To avoid a cross-reactivity of these available Fc determinants, the sections were dipped

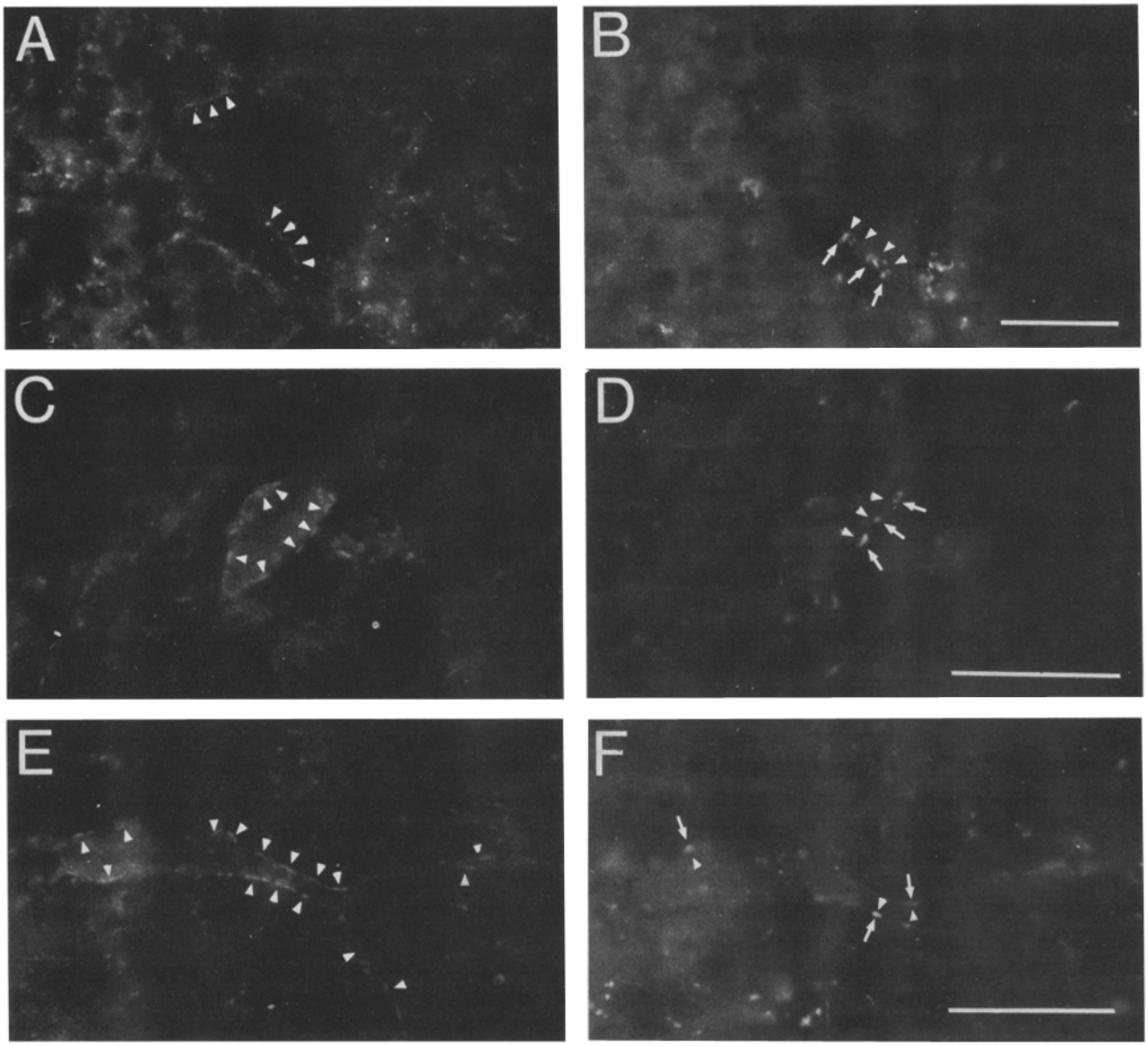


Figure 2. GAD-containing terminals apposed to glycine receptors in lamina VIII and IX of the rat spinal cord after intracardiac perfusion with 4% paraformaldehyde in 0.12 M phosphate buffer. (*A–F*) Double labeling of glycine receptors by mAb GlyR7a, and of GABA terminals by a polyclonal anti-GAD. (*A* and *B*) Photomicrographs of large multipolar cells, presumably motoneurons. (*A*) Discontinuous patches (*arrowheads*) of receptor aggregates at the periphery of a large cell (FITC-selective filters). (*B*) GAD-positive terminals (*arrows*) at the periphery of the same neurons (TRITC-selective filters). The loci of juxtaposed glycine receptors are indicated by arrowheads. (*C–F*) Same mode of representation and indications as for *A* and *B*, showing the proximity of GAD-containing terminals and glycine receptor matrix on a small cell (*C* and *D*) and on dendritic arborization (*E* and *F*). Bars, 10 μm.

in a rabbit anti-mouse at high concentration. Also, in order to verify that the GAM-FITC (which was used as a reactant for GlyR5a and GlyR7a) did not react with the mAb GlyR2b, we have treated the slices sequentially, as described in Materials and Methods. Following this protocol, fluorescent patches were present with the TRITC-selective filter (Fig. 1 *E*) due to a reactivity of the SAM-TRITC with the mAb GlyR2b, but corresponding fluorescence was absent with the FITC optics (Fig. 1 *F*) indicating that, with our procedure, the GAM-FITC did not bind to GlyR2b determinants. Thus, and as expected, mAb GlyR5a and GlyR7a stain cellular elements which are closely associated with the elements recognized by GlyR2b.

GAD-containing Terminals and Glycine Receptors in the Spinal Cord

As indicated, glycine receptors patches were present in high density throughout the gray matter of the spinal cord. This distribution was different from that observed with anti-GAD, which only labeled faintly the ventral horn, with the exception of the motor nuclei (lamina IX) which were slightly more immunoreactive than their surroundings. With double immunofluorescence using GlyR5a and GlyR7a as mAb's against glycine receptors, we found that almost all GAD-positive elements were apposed to glycine receptors, as illustrated in Fig. 2. However, all patches recognized by the mAb

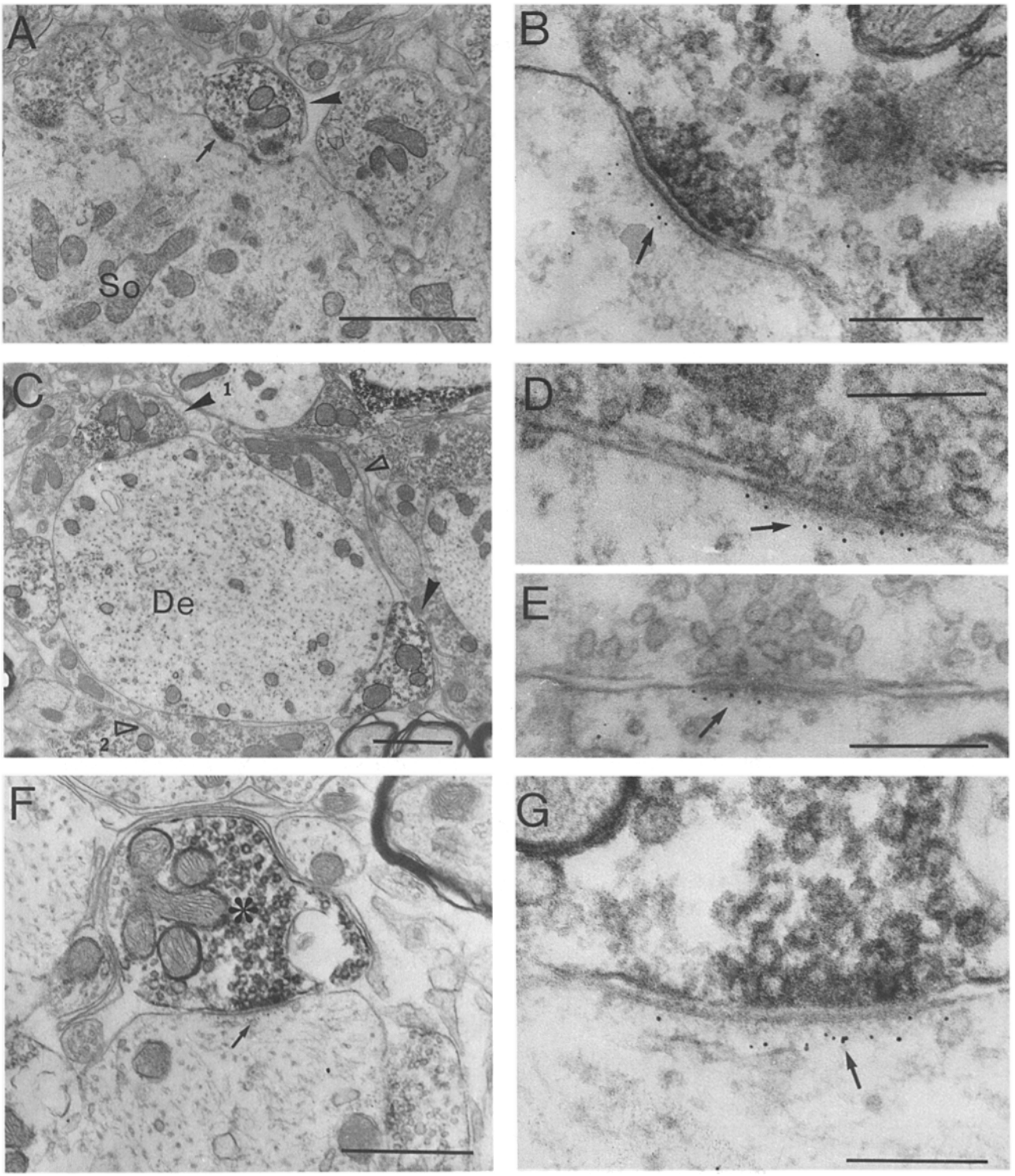
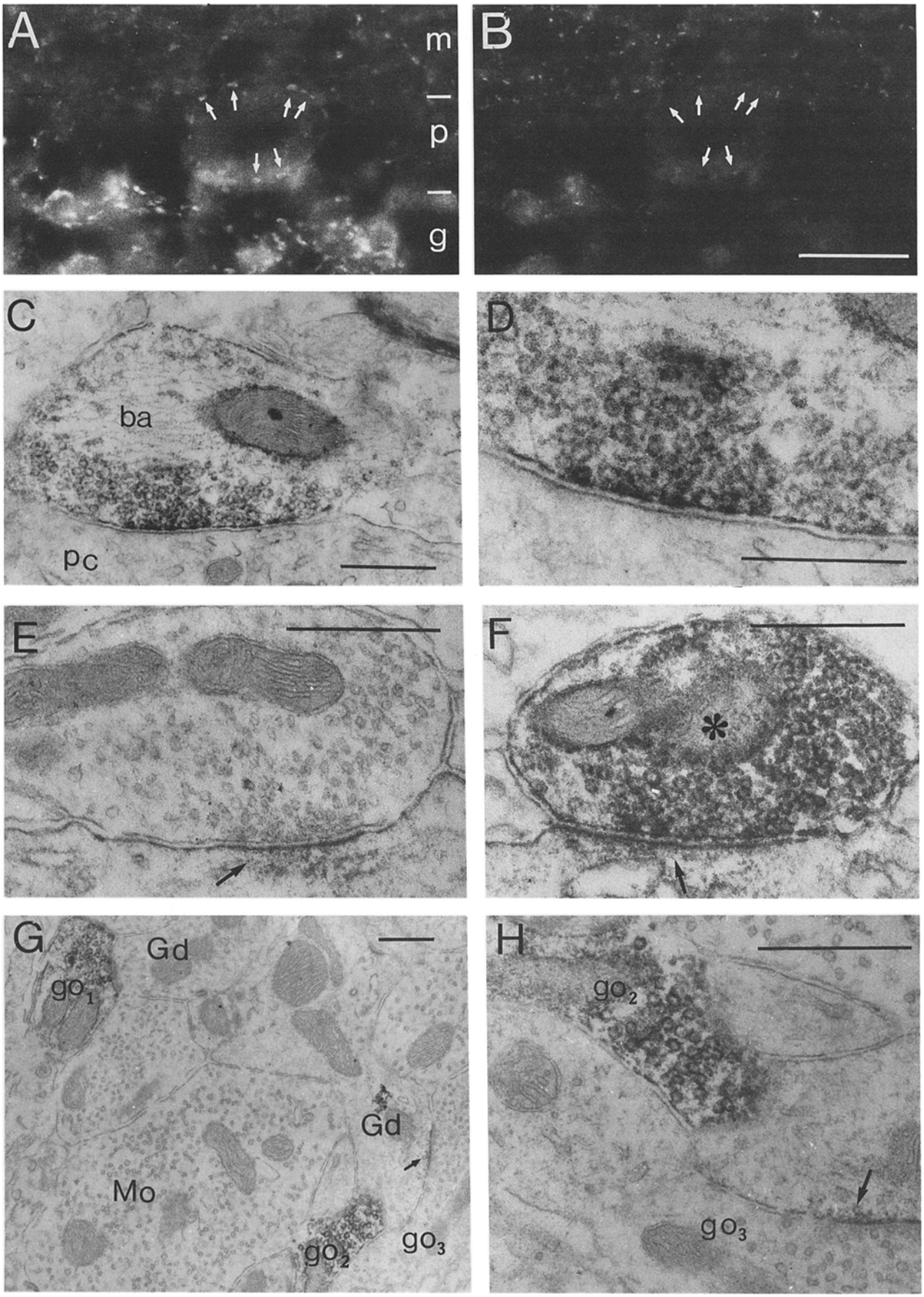


Figure 3. Ultrastructural evidence that GAD-positive boutons can be associated with postsynaptic densities bearing glycine receptor matrices. (*A* and *B*) Low and high power electron micrographs of a presynaptic GAD-positive bouton (*arrowhead*) apposed to a soma (*So*) facing gold particles (*arrows*) linked to mAb GlyR7a. (*C–E*) Micrographs of the same section. (*C*) Contacts established on a dendrite (*De*) by both stained (*arrowheads*) or unstained (*triangles*) boutons. (*D* and *E*) Higher magnifications showing the sites of gold particles linked to mAb GlyR7a (*arrows*). Note that both GAD-positive (*D*) and -negative (*E*) endings, corresponding to boutons labeled *1* and *2*, respectively, in *C*, are apposed to glycine receptor complexes. (*F* and *G*) Same procedure as for *D* and *E*, showing that GAD immunoreactivity (*asterisk*) is also apposed to determinants (*arrows*) for mAb GlyR5a. Fixation procedure: intracardiac perfusion with 4% paraformaldehyde and 0.1% glutaraldehyde in 5 mM phosphate buffer. Bars: (*A* and *C*) 2 μm; (*B, D, E,* and *G*) 0.2 μm; (*F*) 0.5 μm.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were not necessarily in close vicinity of GAD-positive profiles. These conclusions were valid for large cells, presumed to be motoneurons (Fig. 2, *A* and *B*) and for smaller neurons (Fig. 2, *C* and *D*) within the ventral horn. Other processes, most probably dendritic profiles since some clearly arose from recognizable somata, exhibited juxtaposed patches of fluorescence (Fig. 2, *E* and *F*). Identical pattern of labelings were observed with both mAb's used. The proportion of glycine receptor loci in contact with GAD-positive profiles could not be quantified but they did represent only a fraction of their total amount. (It should be noted that controls made without anti-GAD or mAb's totally lacked the corresponding fluorescence.)

This confluence was confirmed at the ultrastructural level, as illustrated by Fig. 3. Anti-GAD immunoreactivity, characterized by the enzymatic reaction product, was present at some presynaptic terminals, which impinged on neuronal somata (Fig. 3, *A* and *B*) and, more often on large and small dendritic profiles (Fig. 3, *C–G*). These endings established symmetrical contacts with their targets and contained a population of pleiomorphic vesicles although some of them were rather filled with apparently round vesicles. In the same sections, antigenic determinants recognized by mAb's GlyR7a and GlyR5a were visualized by the presence of indirectly linked colloidal gold particles which were found at postsynaptic densities in front of both GAD-positive terminals (Fig. 3, *B*, *D*, and *G*) and boutons devoid of GAD reactivity (Fig. 3 *E*). In fact, as already seen with fluorescence, contacts labeled only for glycine were more numerous. This excess is most likely representative of the reality, since it cannot be accounted for by a difference in the sensitivity of the immunoreactants: if such was the case, the opposite would have been observed, because the GAD assay, using immunoenzymatic reaction, normally has a high yield which contrasts with the poor penetration of the colloidal gold.

The postsynaptic labeling was consistently present at the inner surface of the plasmalemma (Fig. 3, *B*, *D*, *E*, and *G*). Particles were concentrated at the level of the synaptic complex identified by dense projections, a constant distance between the pre- and postsynaptic membranes, and an electron-dense material within the synaptic cleft (Peters et al., 1976). However, postsynaptic differentiations were not always well preserved (Fig. 3, *B* and *G*), probably due to the hypoosmolarity of the fixative which elutes most of the cytosolic proteins (de Camilli et al., 1983).

Finally, gold particles, whether or not present in front of GAD-positive terminals, were disposed in single rows, at a relatively constant distance from the midportion of the membrane: specifically, 21.9 ± 7 nm (n = 161) and 30.7 ± 10.2 nm (n = 150) for GlyR7a (Fig. 3, *B*, *D*, and *E*) and GlyR5a (Fig. 3 *G*), respectively. These values are identical to those reported previously (Triller et al., 1985). In other words, no significant structural differences could be detected between synaptic complexes containing GAD-positive or -negative presynaptic elements. The only exception was that the synaptic vesicles seemed rounder in the presence of GAD but this observation may be meaningless since they may have been altered by both the hypoosmotic shock and the decoration of the vesicular membrane by the diaminobenzidine–OsO_4.

Specificity of Glycine Receptor Labeling

To eliminate the possibility that mAb's directed against glycine receptors cross-react with those against GABA, we have examined the relationships between the distribution of GAD and glycine immunoreactivities in the cerebellar cortex. As illustrated at the light microscopic level, GAD was present in the molecular and granular layers and also around the Purkinje cells (Fig. 4 *A*). With glycine receptors mAb's, fluorescence was mostly observed in the molecular layer (Fig. 4 *B*), only few patches were detected in the granular layer, and none around the Purkinje cell somata. These observations were confirmed at the electron microscopic level, using the immunoperoxidase technique for both monoclonal and polyclonal antibodies in order to avoid problems due to the capricious penetration of colloidal gold. In these instances, the enzymatic reaction product observed at pre- or postsynaptic elements necessarily signaled GAD or mAb's, respectively. This conclusion was verified by using one of the antibodies alone.

An important control result was obtained, in this structure, with the double enzymatic approach; namely, that GAD immunoreactivity was only present in the presynaptic element of the basket to Purkinje cell synapses (Fig. 4, *C* and *D*) and that postsynaptic staining was never observed. This not only indicates that antigenic determinants recognized by mAb's GlyR5a and GlyR7a are not expressed at the soma of Purkinje cells but, primarily, confirms that immunoglobulins directed against GAD do not react with the postsynaptic surface. Glycine receptors were, however, detected in other regions of these cells; more specifically, in the molecular layer with reaction deposits being found at the inner membrane surface of the main dendrites (Fig. 4 *E*). All the concerned contacts had the typical aspects of stellate cell syn-

Figure 4. Demonstration that cerebellar neurons can express glycine receptors, and that mAb's directed against them do not cross-react with GABA receptors. All material double-labeled. (*A* and *B*) Different distributions of fluorescence associated with GAD and glycine receptors in the cerebellum after perfusion with 4% paraformaldehyde. (*A*) GAD immunoreactivity at the level of the molecular (*m*), granular (*g*), and Purkinje neuron (*p*) layers and at presumed basket cell terminals (*arrows*). (*B*) Bright spots linked to mAb GlyR7a and mostly restricted to the molecular layer. Note their absence in front of the GAD-positive terminals (corresponding positions in *A* and *B* are indicated by arrows). (*C–H*) Ultrastructural localization of GAD and glycine receptors (recognized by mAb GlyR5a) at various cerebellar synapses after intracardiac perfusion with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.12 M phosphate buffer. (*C–D*) Low and high power electron micrograph of a basket (*ba*) cell axon synapsing on a Purkinje cell (*Pc*) soma. Note that this mAb does not cross-react with GABA receptors since reaction product is only present in the presynaptic bouton. (*E* and *F*) Detection of glycine receptors at synapses between stellate neurons and Purkinje cell dendrites. Postsynaptic differentiations (*arrow*) with glycine receptors at two different contacts, one without (*E*) and one with (*F*) GAD-containing terminal (*asterisk*) apposed to the glycine receptor matrix. (*G* and *H*) Evidence that Golgi axon terminals are either GAD positive or are apposed to glycine receptors. (*G*) Low power electron micrograph of a glomerulus. The granule cell dendrites (*Gd*) at the periphery of the mossy fiber (*Mo*) are contacted by two GAD-positive (go_1, go_2) Golgi axon terminals, and by a negative one (go_3) which is the only one apposed to a stained postsynaptic membrane (*arrow*). (*H*) Higher magnification of go_3. Bars: (*A* and *B*) 20 μm; (*C–H*) 0.5 μm.

apses (Palay and Chan-Palay, 1974) and some of the related terminals, which also contained a pleiomorphic population of vesicles, were GAD positive (Fig. 4 *F*). Thus, and in contrast to the immunocytochemical organization of basket cell synapses, that associated with some stellate cells was the same as in the spinal cord.

Yet another combination of staining patterns was apparent in the granular layer of the cerebellar cortex. There, Golgi axon terminals establish inhibitory junctions with granule cell dendrites (Palay and Chan-Palay, 1974). At this level, presynaptic boutons were, as expected, identified at this level on the basis of their pleiomorphic vesicular population as Golgi axon terminals (Palay and Chan-Palay, 1974). As illustrated in Fig. 4 (*G* and *H*), the staining at these contacts was either pre- or postsynaptic, but did not involve simultaneously the two together. This selectivity was probably not due to a differential penetration of the antibodies, since the immunoreactive postsynaptic density facing a nonreactive Golgi terminal could very well be close to a GAD-positive one (Fig. 4 *H*).

Discussion

In the ventral horn of the spinal cord, glycine receptors were shown to be concentrated in front of terminals, some of which contained GAD, an enzyme responsible for the synthesis of GABA. Furthermore, glycine receptors were found in a structure where their presence had not been suspected, and in various combinations with GAD-positive and -negative terminals. For this study, GAD was chosen as the probe for GABAergic terminals in preference to other ligands, such as muscimol (Chan-Palay and Palay, 1978) or benzodiazepines (Mohler et al., 1980; Richards and Mohler, 1984) which can only be used for autoradiography, or to mAb's against the GABA receptor (Schoch et al., 1985) which do not match the requirements for electron microscopy.

Nature of Immunoreactive Material

Glutamic acid decarboxylase is the enzyme responsible for the conversion into GABA of L-glutamic acid, which is readily available from glucose via the tricarboxylic acid cycle (references in Triggle and Triggle, 1976). On the other hand, GAD is not required for the synthesis of glycine, which is a monocarboxylic amino acid having also its source from glucose, but via 3-phosphoglyceric acid and serine (references in Triggle and Triggle, 1976). Thus, GAD immunoreactivity, which closely parallels labeling with anti-GABA (Ottersen and Storm-Mathisen, 1984), clearly distinguishes between the two biosynthetic pathways for the two transmitters and is a reliable indicator of the GABAergic nature of neurons (Oertel et al., 1984). On the other hand, anti-glycine receptor immunoglobulins are the only immunoreactant available for the detection of the glycine receptor (Triller et al., 1985) and allows one to infer that a labeled junction is glycinergic. Such is the case, at identified synapses, of the teleost Mauthner cell (Nakajima, 1974; Triller and Korn, 1981, 1982) which produces a powerful inhibitory postsynaptic potential presumed to be mediated by glycine (Faber and Korn, 1980): in confirmation with these mAb's, immunoenzymatic reaction products were found in front of the active zones (Triller et al., 1986).

The double labeling EM experiments used mAb's GlyR5a and GlyR7a which recognize the 93-kD polypeptide of the glycine receptor (Pfeiffer et al., 1984) but not mAb GlyR2b which binds to a subunit bearing the antagonist binding site (Pfieffer et al., 1984), since GAD is a soluble enzyme and is lost during the process of fixation needed to stain with the latter (Triller et al., 1985). This was not a problem, since we were able to demonstrate, at the light microscopic level, the association of the determinants that all three mAb's recognize and since, furthermore, the 93-kD subunit co-purifies with the 48-kD protomer of the receptor of different mammalian species (references in Graham et al., 1985): it is, however, a peripheral membrane protein located at cytoplasmic domain of the glycine receptor which can be separated from the strychnine-binding site (Schmitt, B., and H. Betz, personal communication). The possibility that mAb's directed against the glycine receptor cross-react with GABA receptors was checked in the cerebellar cortex, first at the basket to Purkinje cell synapses, which were presumed to be purely GABAergic (Bitsi et al., 1971). No labeling was observed in front of these terminals which were, again, stained with anti-GAD. The lack of correspondence between immunoreactivity to mAb's and GAD was further strengthened by the fact that, conversely, Purkinje cells dendrites express glycine receptor immunoreactivity in front of GAD-positive axons while, in the granular layer, the two systems do not overlap. This last result is consistent with the view that there are two populations of Golgi neurons, transporting glycine or GABA (Wilkin et al., 1981) and with reports of a calcium-dependent efflux of glycine observed in slices (Toggenburger et al., 1983). These results were strengthened by the fact that the mAb's used for this work did not cross-react on immunoblots with the GABA receptor subunits (Schmitt, B., and A. Stephenson, personal communication). Indeed, the 93-kD peptides recognized by the specific mAb's might be linked in a tissue-specific manner to other integral membrane proteins, particularly in relation with GABA receptors. That this is unlikely is suggested by the above results indicating that immunoreactivity can be detected in front of GAD-positive terminals at the level of Purkinje cell dendrites, but not at the somatic region of the same type of neurons facing GABAergic terminals. It still could be, however, that the same mAb's cross-react with receptors for other amino acids.

Colocalization of Classical Inhibitory Transmitters and Possible Functions

There is growing evidence that in the central nervous system, neuronal terminals release more than one substance when activated (see Ljunberg and Hökfelt, 1983). In most cases, "classical" transmitters coexist with peptides (Hökfelt et al., 1980). Systems that use GABA have been shown to contain motilin in the cerebellum (Chan-Palay et al., 1981), somatostatin in the thalamic reticular nucleus (Oertel et al., 1983), and cholecystokinin in the hippocampus and visual cortex (Somogyi et al., 1984). Because their role is not always clear, these associated molecules have been termed neuromodulators. However, GAD was found to coexist with more classical mediators, such as serotonin (Belin et al., 1981) in neurons of the dorsal raphe nuclei, histamine (Takeda et al., 1984) in neurons of the posterior hypothalamic, or catecholamines (Kosaka et al., 1985) in the magnocellular nucleus of the olfactory bulb.

Various hypotheses could account for the presence of these molecules together. It is not impossible that GABA acts presynaptically (see references in Brennan, 1982) to control reuptake and/or release. More intriguing is that both could act postsynaptically, with at least two possibilities, both consistent with evidence that single neurons are sensitive to iontophoretic applications for both glycine and GABA, such as the Mauthner cell (Diamond and Roper, 1973; Diamond et al., 1973) or spinal motoneurons (Barker and McBurney, 1979). One is that GABA and glycine bind with their specific receptors known to produce an increase in Cl^- conductance (Krjnevic and Schwartz, 1967; Werman et al., 1968; Curtis et al., 1968; Faber and Korn, 1980), whether these receptive matrices are intermingled in a mosaic-like fashion or clearly separated with, for example, those for glycine facing the presynaptic active zone and those for GABA being displaced peripherally. The second option is that GABA interacts with the glycine receptors. For instance, it has been suggested that at the level of the Mauthner cell of teleost, GABA can modify allosterically the glycine receptors so as to increase its affinity for glycine (Werman, 1980). Indeed, it is possible that GABA has a dual effect, both on its own and on the glycine receptors as well. Such duplicity could account for the postulated nonlinear processing of inputs from neighboring synapses (Korn and Mallet, 1984) or for heterosynaptic interactions (Changeux et al., 1984; Changeux, 1986). However, the report of a cross-desensitization between GABA and glycine at mouse spinal neurons (Barker and McBurney, 1979) has not been confirmed by others (see Nelson et al., 1977). In fact, the considerable wealth of physiological information already available does not permit a distinction between these two alternatives, both of which would be compatible with the fact that both GABA and glycine activate multistate chloride channels, with different dominant conductances (Hamill et al., 1983), with a higher affinity of the receptor for GABA (Ticku et al., 1980) than of that for glycine (Graham et al., 1985), and with a distinct pharmacology (references in Triggle and Triggle, 1976).

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